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ENHANCED ANGIOGENESIS FOR TISSUE REGENERATION USING HUMAN STEM CELLS AND BIODEGRADABLE NANOPARTICULATE POLYMERIC VECTORS

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INTRODUCTION

Mesenchymal stem cells (MSC) are progenitor cells which can differentiate down multiple lineages including bone. Ideally, scaffold for bone tissue engineering should exhibit biofunctionality of natural materials. In this regards, a novel polymeric biocomposite hydrogel was synthesized by covalently binding of polyethylene glycol (PEG) The most common injuries in battle fields are amputated limbs and massive loss of craniofacial tissues. Current therapies for traumatic tissue injury utilize non-biological and non-degradable materials, which could not provide satisfactory long-term biological function recovery. Angiogenesis is a crucial step for tissue regeneration and integration with the host tissue for function recovery. Vascular endothelial growth factor (VEGF) is a positive regulator and plays a crucial role in angiogenesis.

Stem cells have received significant attention as the next generation therapeutics for regenerative medicine [1]. Using stem cells as delivery vehicles for VEGF holds great promise to enhance angiogenesis and tissue regeneration. The major barrier to gene delivery to stem cells is the lack of safe and efficient DNA delivery methods. Viral methods are high efficient in transferring DNA to the stem cells, but are limited by potential toxicity and immunogenicity [2]. Non-viral systems can circumvent these problems but suffer from low transfection efficiency [3]. Our lab developed a large library of 2350 structurally unique poly(β-amino esters) using combinatorial chemistry [4]. High-throughput screening has shown PBAEs that are promising for non-viral gene delivery due to their ability to condense DNA into small size nanoparticles, low cytotoxicity, and biodegradability [5, 6].

The aims of this work are two fold: 1. To develop an effective and safe biodegradable polymeric vector system for VEGF delivery to human adult and embryonic stem cells. 2. To develop these methods for the purposes of enhancing angiogenesis *in vivo*.

METHODS

Polymer Synthesis:

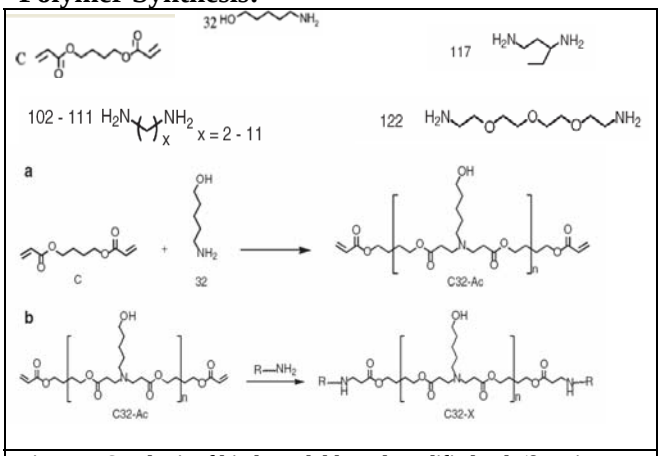

Figure 1. Synthesis of biodegradable end-modified poly(β-amino esters). Acrylate Esters and Amine Monomers used for synthesis were shown . Synthesis of acrylate-terminated C32 polymer (C32-Ac) was first performed by 1.2:1.0 diacrylate: amine polymerization. End-modification of acrylate-terminated C32 with different amine molecules.

Cell Culture: Bone marrow-derived hMSCs (Lonza, Walkswille, MD) were grown in MSC growth medium consisting of Dulcecco's modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco). Cells were subcultured upon confluence until passage 5. Human embryoinc stem cells (H9) were expanded on MEF and then induced to form EBs. Day 8 EBs were dissociated and transferred to gelatin-coated plates. The derived hESCd cells were subcultured in MSC growth medium until passage 2 before use.

Transfections: The promising vectors were optimized for high efficacy and low cytotoxicity to human MSCs and hESCds in growth medium containing 10% serum. For the screening purpose, cells were transfected with pEGFP plasmid and results were analyzed by flow cytometry. Vector parameters were varied including polymer type, polymer/DNA weight ratio (20, 30, 40), and DNA loading dosage (3ug or 6ug/well in a 24-well plate). For angiogenesis study, cells were transfected with VEGF plasmid using lead polymeric vectors. Cells were also transfected using lipofectamine 2000, a leading commercially available transfection reagent

as control. Polyplexes were formed by mixing poly(b-amino esters) with DNA in sodium acetate buffer and waiting 10 min for the complexes to form. The polyplexes were then added to cells cultured in growth medium containing 10% serum and incubated for 4 hours. Following a 4 hr transfection incubation time, the complexes were removed and refilled with fresh growth medium.

Flow Cytometry: Seventy-two hours post-transfection, GFP expression was measured using fluorescence activated cell sorting (FACS) on a FACSCalibur (Becton Dickinson). Propidium iodide staining was used to exclude dead cells from the analysis and 10,000 live cells per sample were acquired.

VEGF Production: VEGF secretion was measured using a VEGF ELISA kit (R&D Systems). Briefly, medium from transfected stem cells was collected at multiple time points and assayed for ELISA. Untreated cells or cells transfected using lipofectamine 2000 were included as controls.

***In vivo* Angiogenesis**: Cells overexpressing VEGF were seeded in three-dimensional biodegradable PLGA/PLLA scaffolds and implanted subcutaneously in athymic mice. Tissue constructs were harvested two weeks and three weeks post-implantation. Outcome was measured by histology and image analyses.

RESULTS

High Transfection Efficiency

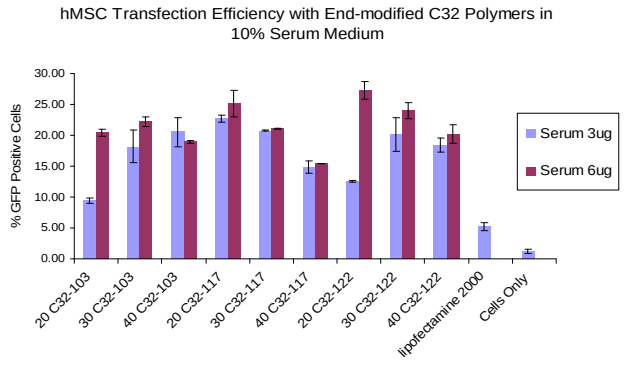

Figure 2. FACS transfection results of passage 5 hMSCs in 10% serum media. Under optimal conditions, lead end-modified C32 polymers showed ~ 5 fold higher transfection efficiency compared to Lipofectamine 2000. Error bars are standard deviations (n=4).

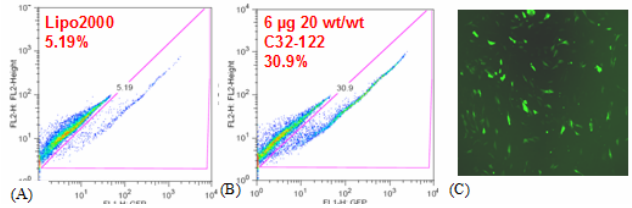

Figure 3. Two-dimensional density plot of hMSC FACS data of (A)lipofectamine 2000, (B) C32-122. (C) Fluorescence images of hMSCs transfected by C32-122

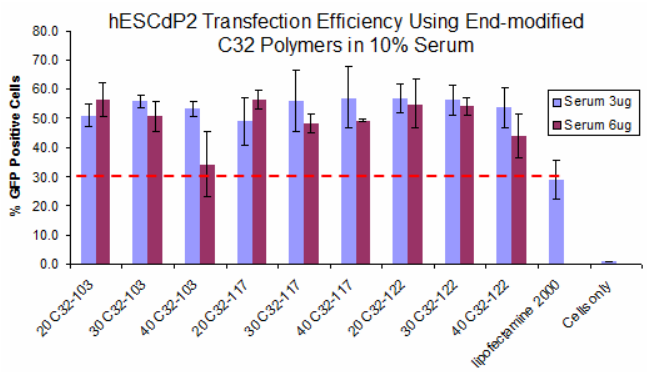

Figure 4. FACS results of passage 2 hESCd cells in 10% serum media. End modified C32 polymers demonstrated ~100% higher transfection efficiency compared to Lipofectamine 2000. Error bars are standard deviations (n=4).

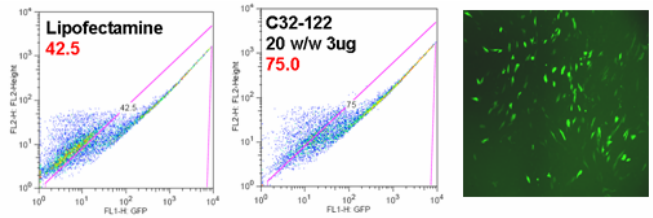

Figure 5. Two-dimensional density plot of hESCd FACS data of (A) lipofectamine 2000, (B) C32-122. (C) Fluorescence images of hESCd cells transfected by C32-122.

Fig 3. Von Kossa staining and Immunohistochemical staining of Col I and Col II by three weeks in vitro culture.

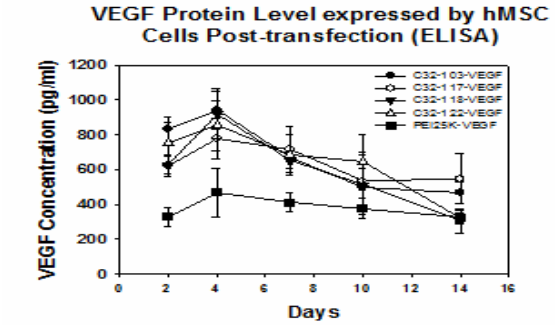

Figure 6. ELISA results showed that human mesenchymal stem cells (hMSC) secreted a base level of VEGF. Compared to the controls (PEI). Groups transfected using end-modified C32 polymers led to ~2 fold higher VEGF production in hMSCs . The upregulation of VEGF secretion reached its peak around day 4 and then gradually came back to the base line by 2 weeks.

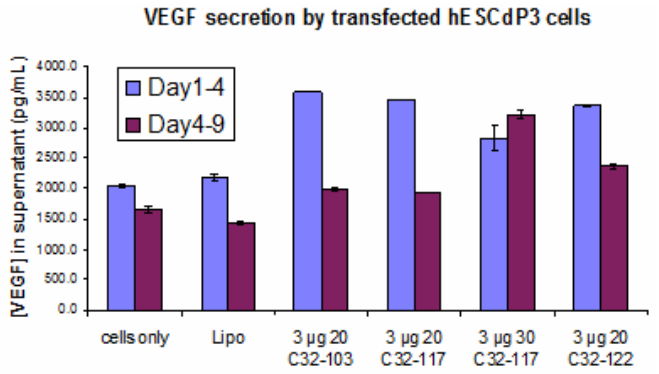

Figure 7. Human ESC-derived cells (hESCds) secreted a base level of VEGF. Compared to the controls (PEI or Lipofectamine), groups transfected using end-modified C32 polymers led to 1 fold higher VEGF production in hESCds cells. The upregulation of VEGF secretion reached its peak around day 4 and then gradually came back to the base line after 9 days.

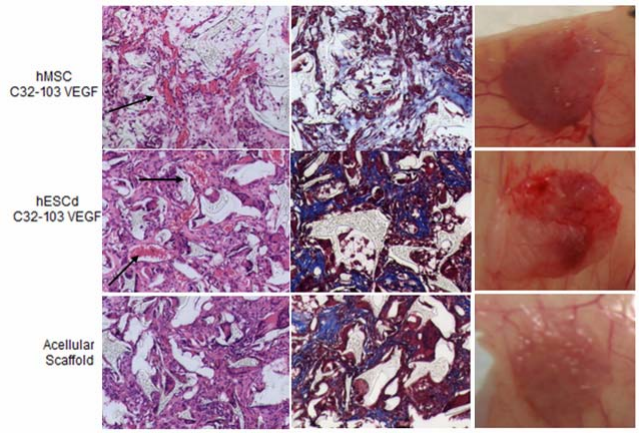

Figure 8. Two weeks after transplantation in subcutaneous space in athymic mice, scaffolds seeded with stem cells overexpressing VEGF demonstrated significantly enhanced angiogenesis (arrows) compared to the acellular controls, as shown by both histology (H&E and Masson's Trichrome staining) and gross appearance.

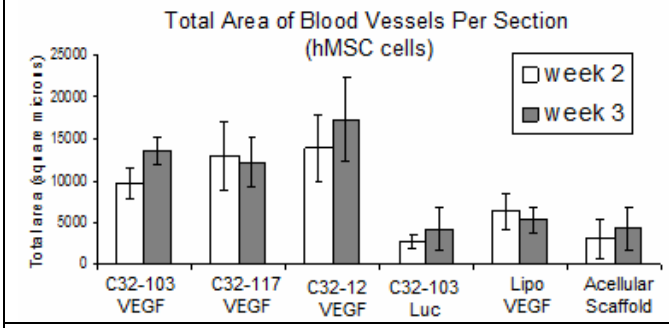

Figure 9. Image analyses of total area of blood vessels per section by human mesenchymal stem cells (hMSCs). Groups transfected with VEGF using lead end-modified C32 polymers led to 2-3 fold higher of total area of blood vessels compared to the acellular scaffold control.

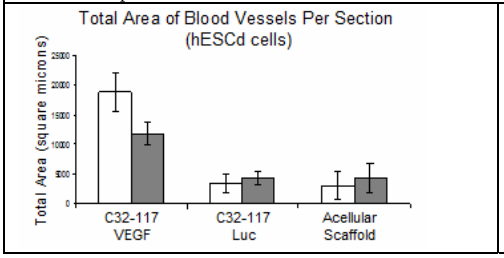

Figure 10. hESCd cells transfected with C32-117 polymer and VEGF plasmid induced significant angiogenesis compared to the controls.

CONCLUSIONS

We have demonstrated that poly(-amino esters), and end-modified C32 polymers in particular, are highly efficient vectors for VEGF delivery to both adult and embryonic-derived stem cells.

These results suggest that combined approach of stem cell and gene therapy using biodegradable polymers can provide an effective system to promote therapeutic angiogenesis *in vivo*, both for the purpose of tissue regeneration and for the salvage of ischemic limb. As angiogenesis is an antecedent crucial step for most types of tissue regeneration, this technology will have broad applications for repair of massive tissue loss incurred in battle fields.

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